

Rec'd PCT/PTO 08 SEP 2004

10/506962

PCT/EP 03/02361

#2



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

REC'D 17 AFR 2003

WIPO

PCT

Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

0.2005452.4

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

BEST AVAILABLE COPY

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Europäisches
Patentamt

European
Patent Office

PCT/EP 03/02361

Office européen
des brevets

Anmeldung Nr:
Application no.: 02005452.4
Demande no:

Anmeldetag:
Date of filing: 08.03.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Max-Planck-Gesellschaft zur Förderung der
Wissenschaften e.V. Berlin
Hofgartenstrasse 8
80539 München
ALLEMAGNE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se référer à la description.)

Use of EGFR transactivation inhibitor in human cancer

In Anspruch genomene Priorität(en) / Priority(ies) claimed / Priorité(s)
revendiquée(s)

Staat/Tag/Aktenzeichen/State/Date/File no./Pay /Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

A61K39/00

Am Anmeldetag benannte Vertragsstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

WEICKMANN & WEICKMANN

Patentanwälte
European Patent Attorney · European Trademark Attorneys

08. März 2002

EPO - Munich
20

08. März 2002

DIPL.-ING. H. WEICKMANN (bis 31.1.01)
DIPL.-ING. F. A. WEICKMANN
DIPL.-CHEM. B. HUBER
DR.-ING. H. LISK
DIPL.-PHYS. DR. J. FRECHTEL
DIPL.-CHEM. DR. B. BOHM
DIPL.-CHEM. DR. W. WEISS
DIPL.-PHYS. DR. J. TIESMEYER
DIPL.-PHYS. DR. M. HERZOG
DIPL.-PHYS. B. RUTTENSBERGER
DIPL.-PHYS. DR.-ING. V. JORDAN
DIPL.-CHEM. DR. M. DEY
DIPL.-FORSTW. DR. J. LACHNIT

Our Ref:
27675P EP/WWmypo

Applicant:
Max-Planck-Gesellschaft zur
Förderung der Wissenschaften e.V.
Hofgartenstraße 8

80539 München

Use of EGFR transactivation inhibitors in human cancer

Use of EGFR transactivation inhibitors in human cancer

Description

5

The present invention relates to the use of a compound which is capable of inhibiting activation of growth-factor receptors of the EGFR-family for the prevention or treatment of processes associated with increased G-protein mediated signal transduction.

10

Signaling through receptor tyrosine kinases (RTK) is involved in the regulation of fundamentally important cellular processes and its upregulation has been shown to be connected to hyperproliferative diseases such as cancer. G protein-coupled-receptors (GPCR) constitute the largest group of cell surface receptors controlling multiple signaling cascades and their biological outcome. Recently, crosstalk between members of both receptor families has been described that connects the multitude of different stimuli via GPCR ligands with the signaling capability of RTKs such as the epidermal growth factor receptor (EGFR), see e.g. WO01/12182. The signaling mechanism which involves shedding of growth-factor precursors by a metalloprotease led us to propose the model of the triple-membrane-passing signal (TMPS) pathway.

15

20

Our object was to investigate the physiological processes that are regulated by the TMPS pathway and its significance for pathophysiological phenomena such as neoplastic progression. Therefore, we screened human cancer cell lines for EGFR transactivation by stimulation with GPCR ligands, as well as inhibition of metalloproteases. Specifically, we investigated growth factor-stimulated events in signal transduction and in defined physiological processes.

25

30

Our results show that EGFR phosphorylation as well as downstream signaling events such as the recruitment of adapter proteins and phosphorylation of the mitogen-activated protein kinase occur after stimulation with GPCR ligands and are downregulated by the metalloprotease inhibitor batimastat. Additionally, basal phosphorylation of the EGFR is batimastat sensitive. Furthermore, we revealed that EGFR transactivation is part of the regulatory system which modulates cell cycle progression and cell proliferation. The TMPS pathway is also able to promote anti-apoptosis, cell migration and invasivity.

While somatic cells require external mitogenic signals, cancer cells are characterized by abnormal growth behaviour due to autocrine production of mitogenic factors. As many of these are GPCR ligands, EGFR transactivation constitutes a mechanism for cancer progression by deregulation of cell proliferation and suppression of cell death. Inhibition of this pathway is therefore a promising strategy for the treatment of cancer.

Thus, a subject matter of the present invention is the use of a compound which is capable of inhibiting activation of a growth-factor receptor of the EGFR family for the manufacture of an agent for the prevention or treatment of processes selected from cell proliferation, cell migration, invasivity and anti-apoptosis in a disorder, which is associated with increased G-protein mediated signal transduction.

Surprisingly, it was found that inhibition of growth-factor receptor activation caused by increased G-protein mediated signal transduction leads to an inhibition of cancer progression, particularly cell migration and invasivity, as well as to an inhibition of anti-apoptosis. Thus, inhibitors of the GPCR-induced growth-factor receptor activation are suitable for the manufacture of medicaments for the prevention or treatment of specific indication of hyper-proliferative diseases associated with cell-proliferation,

cell migration, invasivity and/or anti-apoptosis and to re-establish control of these phenomena in the treated cells or organisms, respectively.

5 The growth-factor receptor is preferably EGFR or another member of the EGFR family, such as HER-2, HER-3 or HER-4, but also other growth-factor receptors, particularly receptor tyrosine kinases may be inhibited.

10 The compound may act on a growth-factor receptor ligand precursor, which is preferably a membrane-associated molecule. In a particularly preferred embodiment the growth-factor ligand precursor is pro-HB-EGF which is cleaved to HB-EGF by a protease. Other preferred examples of growth-factor receptor ligands which are cleaved from precursors are other members of the EGF family, such as TGF α , amphiregulin, epi-regulin, EGF, β -cellulin and members of the heregulin/NDP family including isoforms thereof.

15 An example of a compound, which acts on a growth-factor receptor ligand precursor, is CRM197, a catalytically inactive form of the diphtheria toxin, which specifically binds to pro-HB-EGF and which is capable of blocking the processing of pro-HB-EGF. A further example is an antibody, which is capable of binding to pro-HB-EGF and which thereby blocks its processing.

20 In a further embodiment, the compound acts on a metalloprotease, particularly a membrane-associated metalloprotease, e.g. a protease of the ADAM family. Inhibition of the protease leads to a blocking of the processing of growth-factor receptor ligand precursors and thus results in an inhibition of growth-factor receptor activation. Preferred examples of inhibitors of protease activity are batimastat (BB-94), marimastat, TAPI and TIMP-1-2-3 or -4, particularly TIMP-3.

30 In a still further embodiment the compound may act on the growth-factor receptor itself. For example, the compound may bind to the growth-factor

receptor and thereby inhibit transactivation. An example of such a compound is the EGFR-inhibitor AG1478. Further, growth-factor receptor-binding antibodies may be used.

- 5 It should be noted that the present invention relates to a targeted inhibition of cellular signal pathways "downstream" of the EGFR transactivation in cancer cells, particularly in human cancer cells.

10 The disorder to be treated or prevented is associated with and preferably caused by increased G protein-mediated signal transduction. This increased G protein-mediated signal transduction may be associated with or caused by a pathological increase in the activity of a G protein and/or a G protein-coupled receptor (GPCR). It should be noted that the disorders which are prevented or treated according to the present invention can be delimited
15 from other hyperproliferative disorders having an enhanced growth-factor receptor expression in that a transactivation of growth-factor receptor expression via G protein signal pathway takes place. More particularly, the disorder is a cancer, such as a colon, kidney, liver, bladder, pancreatic, prostate, gastric, breast, lung, thyroid, pituitary, adrenal or ovarian tumor
20 or glioblastoma.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. A therapeutically
25 effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g. for determining the LD₅₀ (the dose lethal to 50% of the
30 population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture

assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 as determined in cell culture (i.e. the concentration of the test compound which achieves a half-maximal inhibition of the growth factor receptor activity). Such information can be used to more accurately determine useful doses in humans. The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds which exhibit high therapeutic indices are preferred. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (see e.g. Fingler et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1, p. 1). Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the receptor modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data, e.g. the concentration necessary to achieve a 50% inhibition of the receptor using the assays described herein. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%. Dosages necessary to achieve these plasma levels will depend on individual characteristics and route of administration. In cases of local administration the concentration of the drug may not be related to plasma concentration.

The actual amount of composition administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration and the judgement of the prescribing physician. For batimastat and other compounds e.g. a daily dosage of 1 to 200 mg/kg, particularly 10 to 100 mg/kg per day is suitable.

Further, the invention relates to a method for identifying and/or characterizing an inhibitor of processes selected from cell proliferation, cell migration, invasivity and anti-apoptosis in a disorder associated with increased G-protein mediated signal transduction, comprising: determining the effect of a test compound on the inactivation of a growth-factor receptor of the EGFR family. This method is preferably a screening method which comprises a functional assay for cell proliferation, cell migration, invasivity and/or anti-apoptosis, e.g. as described in the examples. The test compounds may be selected from low molecular weight compounds, peptides and proteins, particularly antibodies or antibody fragments.

Further, the invention shall be explained in more detail by the following examples:

EXAMPLE

1. METHODS

1.1 Cell lysis, immunoprecipitation and immunoblotting

Prior to lysis, cells grown to 80% confluency were treated with inhibitors and agonists and lysed for 10 min on ice in buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 1% TX-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin. Lysates were prepared by centrifugation at 13000 rpm for 10 min at 4°C. Supernatants were diluted in an equal volume of HNTG buffer and subsequently immunoprecipitated using the respective antibodies and 30 µl of protein A-Sepharose for 4 h at 4°C. Precipitates were washed three times with 0.5 ml of HNTG buffer, suspended in SDS sample buffer and subjected to gel electrophoresis on 7.5% or 10% gels. Following SDS page, proteins were transferred to a nitrocellulose membrane and immunoblotted.

1.2 Invasion assay

Cell invasion assays were performed in medium containing the chemoattractant in the lower well of a Boyden chamber. A Matrigel-coated well of the Boyden chamber and is sequentially preincubated with the inhibitor for 20 min in each well of the chambers in a 24 well plate for 6 h to overnight in a humidified 7% CO₂ incubator. Cells are then wiped from the upper surface with a cotton tip swab and the cells on the lower side are stained and counted.

Boyden chambers. Serumfree medium to be tested is added to the lower well. A filter is placed over the lower well with a gasket. The cells are seeded in the upper well and then added to the upper well. The chambers are incubated in a 37°C incubator. Finally, cells are removed with a cotton tip swab and the cells on the lower side are counted under the microscope.

1.3 Migration assay

Cell migration assays were performed using Boyden chambers containing a polycarbonate membrane (8 µm pores).

Boyden chambers containing a polycarbonate membrane as described above.

1.4 Cell wounding assay

Cells were grown to confluence in six well plates and analysed using a chemical scratch wound assay. Cells were gently scraped with a plastic tip. The medium was removed and the cells were washed twice with PBS. Serumfree Medium was added and the cells were allowed to migrate into the area of the wound. Analysis was performed under the microscope.

Cells were grown to confluence in six well plates in standard medium and analysed using a chemical scratch wound method. Cells were gently scraped with a plastic tip. The medium was removed, and cells were washed twice with PBS. Serumfree Medium was added and the cells were allowed to migrate into the area of the wound for 24 h to 48 h. Analysis was performed under the microscope.

1.5 Thymidine incorporation

Cells were seeded in 96 well plates grown to 70% confluence, and then incubated in serum free medium for 24 h to induce quiescence. Mitogenic stimuli were then added at time 0, and thymidine (1 µCi/well, 3H) was added to each well. After 18 h the cells were harvested and the radioactivity counted.

Cells were seeded in 96 well plates grown to 70% confluence, and then incubated in serum free medium for 24 h to induce quiescence. Mitogenic stimuli were then added at time 0, and thymidine (1 µCi/well, 3H) was added to each well. After 18 h the cells were harvested and the radioactivity counted.

Four hours later the culture media was removed, cells were washed with PBS, fixed with ice cold 10% trichloroacetic acid. Labelled material was then solubilized and dissociated radioactivity was then quantified by liquid scintillation counting.

5

1.6 Detection of Apoptosis

Apoptosis was measured by flow cytometry. Cells were seeded in six well plates and starved. Apoptosis was induced by anti-CD95 antibody, at the same time inhibitors and afterwards in the end of the culture period cells were washed in PBS, resuspended in hypotonic buffer containing 50µg/ml propidium iodide, kept for 2 h at 4°C. The percentage of apoptotic cells was determined by evaluating hypodiploid nuclei after proper gating on DNA content.

15

2. RESULTS

Investigation of the physiological processes that are regulated by EGFR transactivation via the triple-membrane

20

1. Several human cancer cell lines show constitutive activation of EGFR by ligands as well as inhibition of

25

2. Our results show that EGFR phosphorylation as well as downstream signaling events such as the recruitment of adapter proteins and phosphorylation of the mitogen-activated protein kinase occur after stimulation with GPCR ligand. Additionally, basal phosphorylation of the EGFR is

30

removed, cells were washed with PBS, fixed with ice cold 10% trichloroacetic acid. Labelled material was then solubilized and dissociated radioactivity was then quantified by liquid scintillation counting.

Cells were seeded in six well plates and starved. Apoptosis was induced by anti-CD95 antibody, at the same time inhibitors and afterwards in the end of the culture period cells were washed in PBS, resuspended in hypotonic buffer containing 50µg/ml propidium iodide, kept for 2 h at 4°C. The percentage of apoptotic cells was determined by evaluating hypodiploid nuclei after proper gating on DNA content.

that are regulated by EGFR transactivation via the triple-membrane signaling signal (TMPS)-pathway: -

EGFR transactivation by GPCR ligands as well as downstream signaling events such as the recruitment of adapter proteins and phosphorylation of the mitogen-activated protein kinase occur after stimulation with GPCR ligand. Additionally, basal phosphorylation of the EGFR is

phosphorylation as well as downstream signaling events such as the recruitment of adapter proteins and phosphorylation of the mitogen-activated protein kinase occur after stimulation with GPCR ligand. Additionally, basal phosphorylation of the EGFR is

3. EGFR transactivation via the proliferation, cell apoptosis, cell

PS pathway promotes cell ation and invasion.

The results are summarized in Fig. 1-7

in Table 1.

5

3. CONCLUSIONS

10 Cancer cells are characterized by ab
autocrine production of mitogenic fac
ligands, EGFR transactivation constitu
progression by deregulation of cell pr
death. Inhibition of this pathway is the
treatment of cancer.

al growth behaviour due to
As many of these are GPCR
a likely mechanism for cancer
ation and suppression of cell
a promising strategy for the

15

Claim

1. Use of a compound which is capable of inhibiting activation of a growth-factor receptor of the EGFR family for the manufacture of an agent for the prevention or treatment of processes selected from cell proliferation, cell migration, invasivity and anti-apoptosis in a disorder, which is associated with increased G-protein mediated signal transduction.
2. The use of claim 1 wherein the growth-factor receptor is EGFR.
3. The use of claim 1 or 2 wherein the compound acts on a growth-factor receptor ligand precursor.
4. The use of claim 3 wherein the growth-factor receptor ligand precursor is EGF or an EGF-like ligand.
5. The use of claim 1 or 2 wherein the compound acts on a metalloproteinase.
6. The use of claim 5 wherein the compound directly inhibits the protease activity.
7. The use of claim 1 or 2 wherein the compound acts on the growth-factor receptor.
8. The use of any one of claims 1 to 7 wherein the agent is a pharmaceutical composition comprising at least one pharmaceutical acceptable carrier, diluent and/or adjuvant.
9. The use of any one of claims 1 to 3 wherein the disorder is cancer.

10. The use of any one of claims 1 to 9 wherein the cancer is a human cancer.
- 5 11. A method for identifying and/or characterizing an inhibitor of processes selected from cell proliferation, cell migration, invasivity and anti-apoptosis in a disorder associated with increased G-protein mediated signal transduction, comprising:
10 determining the effect of a test compound on the transactivation of a growth-factor receptor of the EGFR family.
12. The method of claim 11, wherein the test compound is selected from low-molecular weight compounds, peptides and proteins, particularly antibodies or antibody fragments.

EPO - Munich
20
08. März 2002

Abstract

The present invention relates to the use of a compound which is capable of
5 inhibiting activation of growth factor receptor of the EGFR-family for the
prevention or treatment of processes associated with increased G-protein
mediated signal transduction.

10 pu/ANM/27675P EP 06 2

Tyrosine phosphorylation of the EGFR and Shc occur after stimulation with GPCR ligands and are downregulated by BB94 and AG1478

FIGURE 1

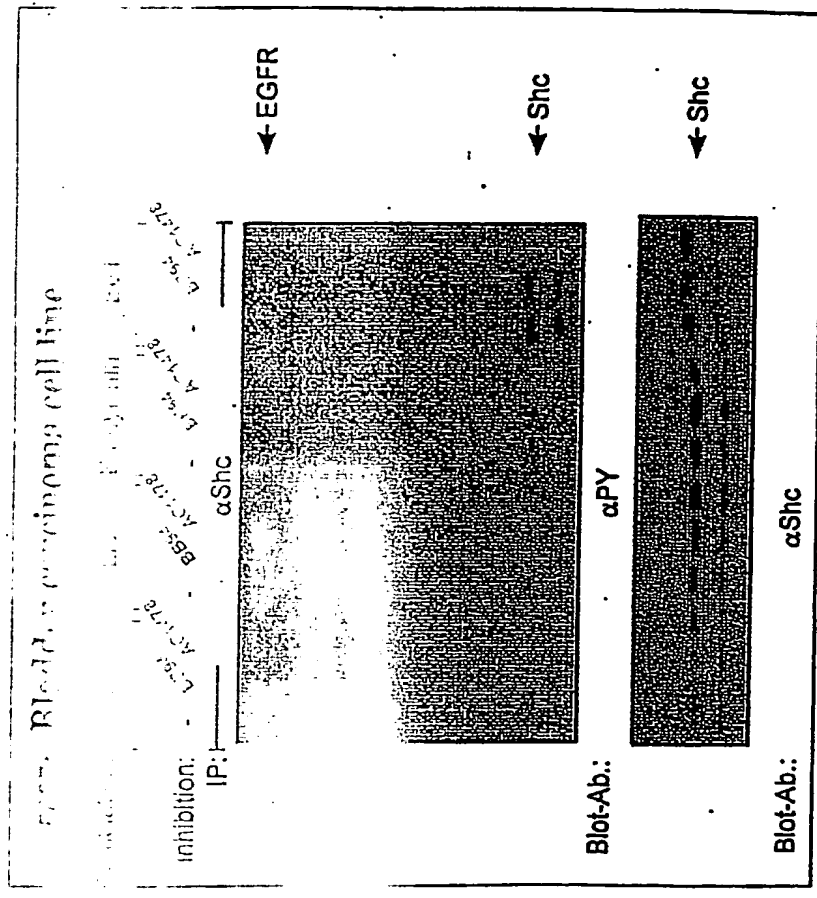


FIGURE 2

Phosphorylation of the mitogen-activated protein kinase after stimulation with GPCR ligands is downregulated by BB94 and AG1478

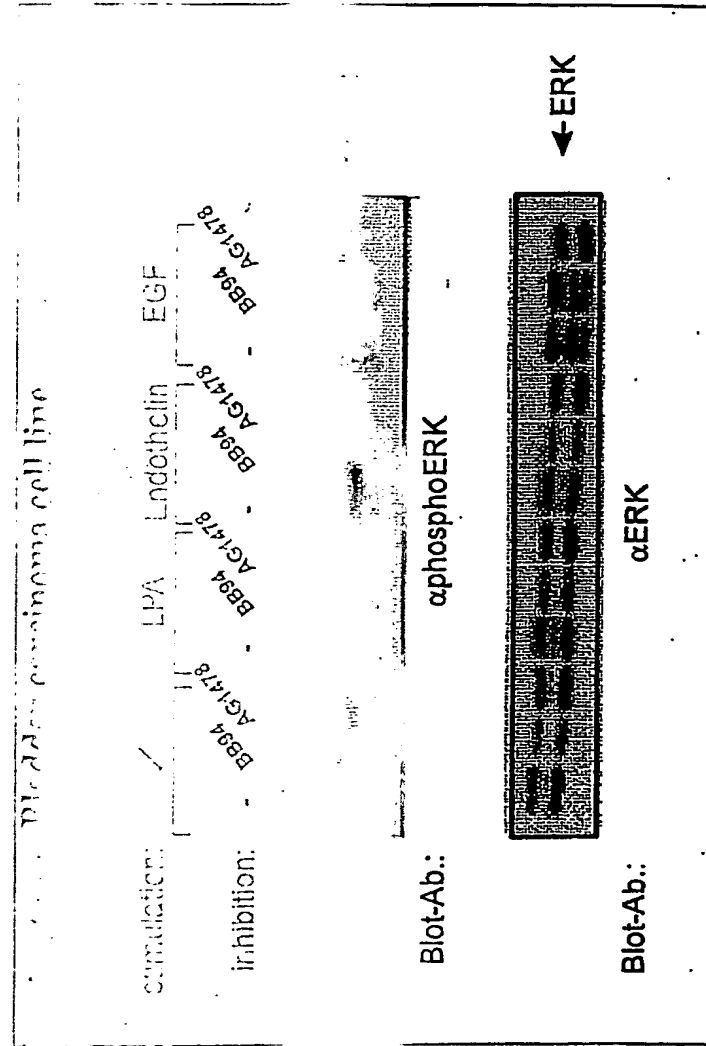
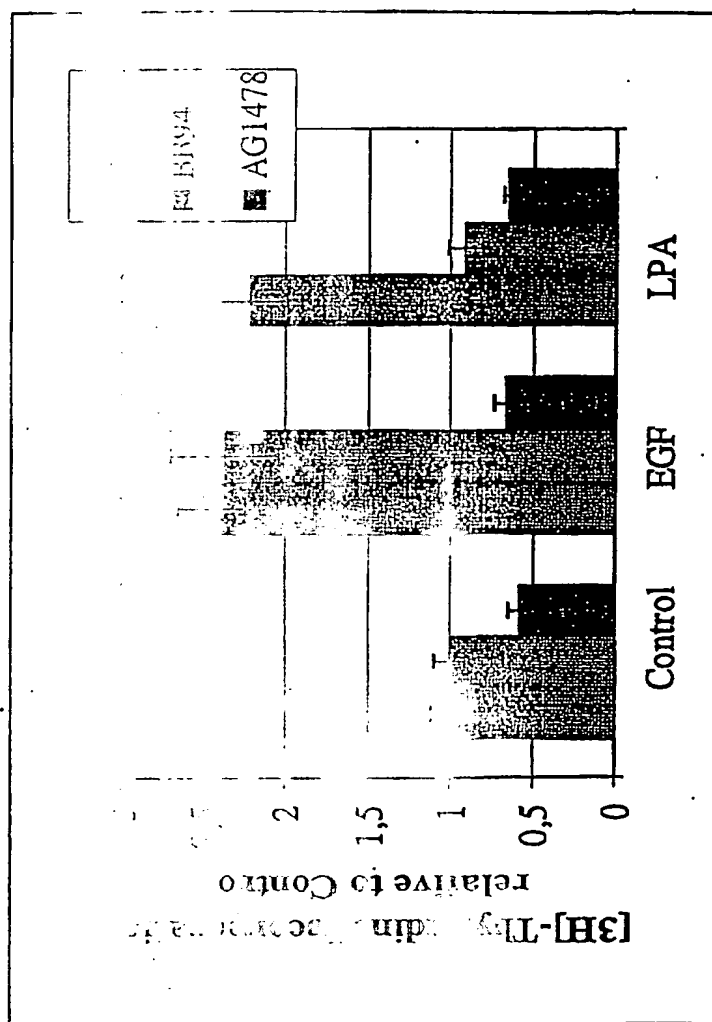


FIGURE 3

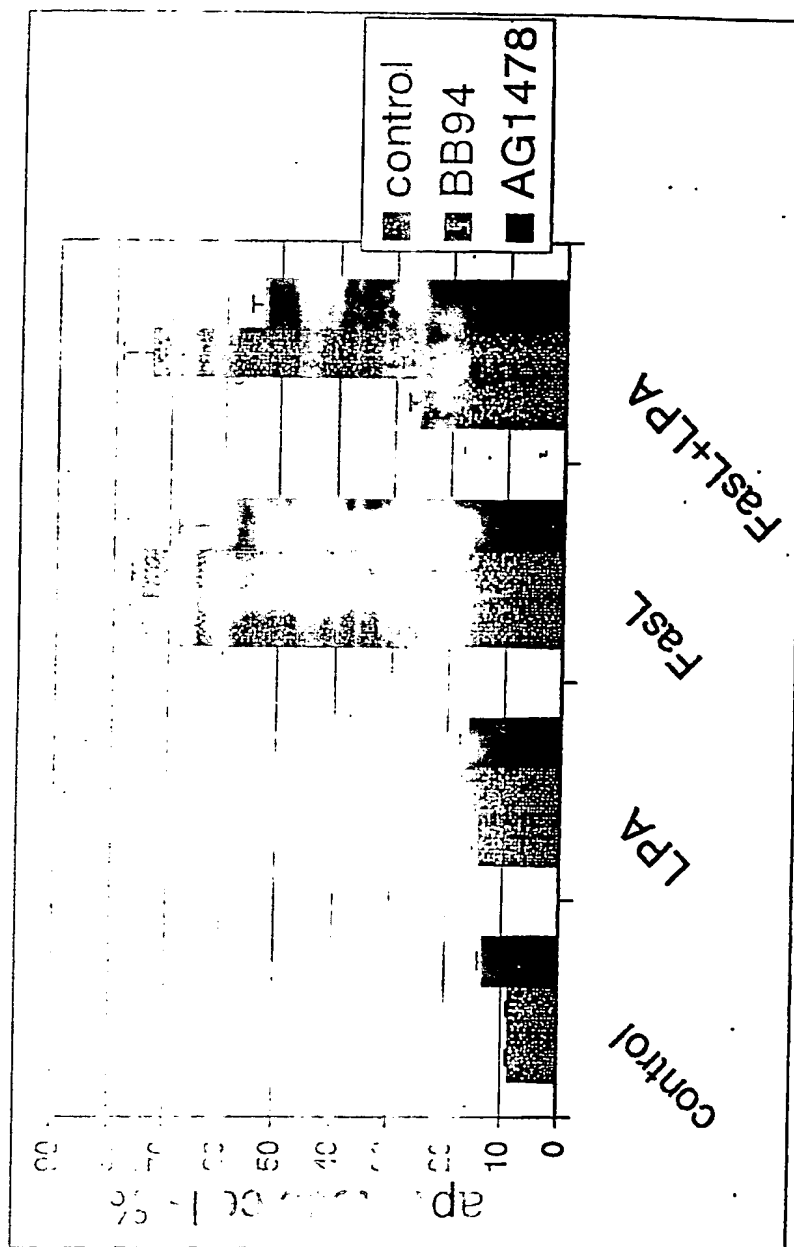
LPA induced proliferation in the lung cancer cell line H292 can be inhibited by BB94 and AG1478



Antiapoptotic effects induced by LPA in the bladder carcinoma cell line TccSup can be inhibited by BB94 and AG1478

FIGURE 4

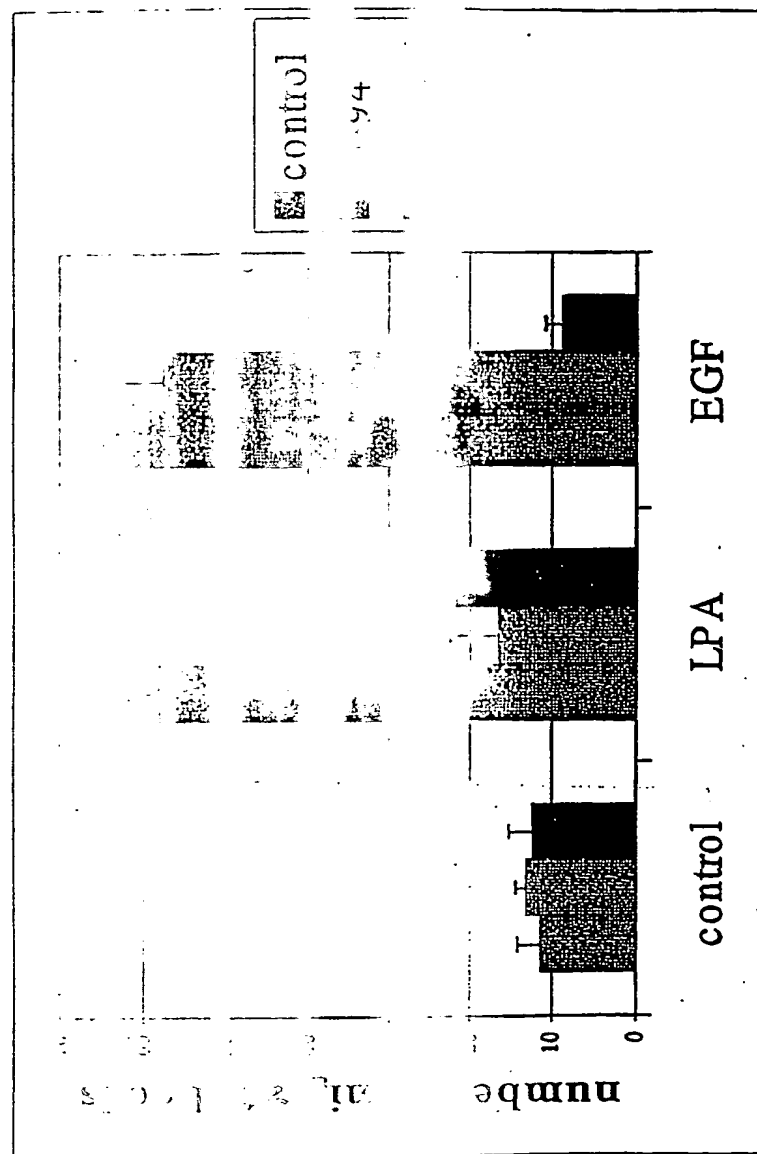
4/8



LPA induced migration in the kidney carcinoma cell line A498 can be inhibited by BB94 and AG1478

FIGURE 5

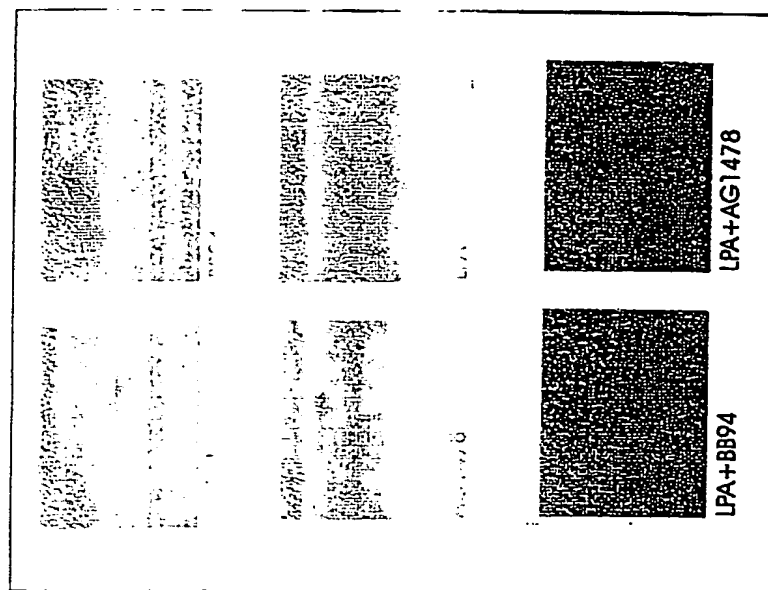
5/8



LPA induced wound closure in the kidney carcinoma
cell line ACHN can be inhibited by BB94 and
AG1478

FIGURE 6

6/8



LPA promotes invasion in the kidney carcinoma cell line CaKi2 which can be diminished by BB94 and

AG1478

FIGURE 7

7/8

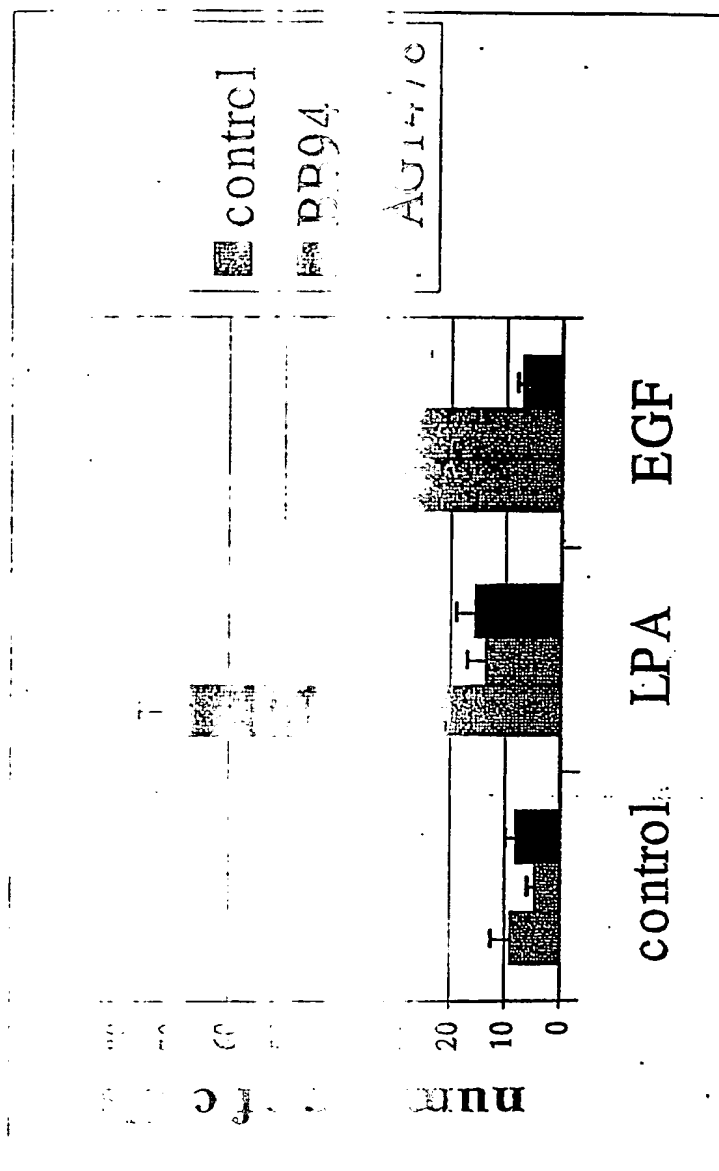


TABLE 1

Screening of human cancer cell lines for the physiological relevance of EGFR transactivation

physiological relevance

proliferation migration invasion antiapoptosis

cell line	human	kidney	n/d	n/d	n/d	n/d	n/d
A498	human	kidney	no	yes	yes	no	n/d
A704	human	kidney	no	n/d	n/d	n/d	n/d
SCA-1	human	bladder	no	no	n/d	no	no
HT-1080	human	bladder	no	no	n/d	no	no
TccSUP	human	bladder	no	yes	yes	yes	yes
H292	human	lung	yes	no	no	no	no

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.